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Research article

Insulin-related signaling pathways elicited by light in photoreceptor nuclei from bovine retina

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ABSTRACT

Retina light stimulation triggers phototransduction events as well as different signaling mechanisms in outer segments (sensorial portion) of photoreceptor cells. We have recently reported a novel light-dependent activation of diacylglycerol kinase (DAGK) and protein kinase C (PKC) at the nuclear level of photoreceptor cells. The aim of the present study was to analyze whether *ex-vivo* light exposure of bovine retinas also modulates insulin-related signaling pathways in nuclei from photoreceptor cells. To this end, a nuclear fraction enriched in small nuclei from photoreceptor cells (PNF) was obtained using a modified nuclear isolation protocol. In PNF obtained from bovine retinas exposed to light or darkness, the presence of insulin receptor (IR) and phosphorylated insulin receptor (pIR), the activation of Akt, p38 and extracellular signal-regulated kinase (ERK1/2) and the local action of insulin on lipid kinases were studied.

Immunofluorescence (IF) and Western blot (WB) studies revealed the presence of IR in photoreceptor nuclei. In PNF a light-dependent increase in IR total content was observed. The presence of activated IR (pIR) was also observed in PNF by WB, being its content higher in PNF from light than in to darkness. Light exposure also produced a significant increase in the content of p-Akt (3 fold) and p-p38 (60%) without changes in total Akt and p38. In addition, an increase in the content of total ERK1/2 (2 fold) was found without changes in p-ERK/total ERK ratio, indicating that light induces translocation of p-ERK to the nucleus.

Polyphosphoinositide kinase and diacylglycerol kinase (DAGK) activities were measured in isolated nuclei from light-activated or darkness-adapted retinas through the formation of polyphosphoinositides (PPIs) and phosphatidic acid (PA) using nuclear lipid substrates and [γ-^{32}P]ATP as radioactive substrate. A light-dependent increase in PPIs and PA formation was detected when isolated nuclei were exposed to 0.8 μM insulin plus 0.2 mM vanadate.

WB studies revealed that retina's exposure to insulin under light condition increased nuclear IR content. In addition, PNF exposure to insulin increased ERK1/2 phosphorylation with no changes in total ERK1/2.

Our results demonstrate the presence and the functional state of IR in the nucleus from photoreceptor cells. They also show that molecular signaling components linked to tyrosine kinase receptors and MAPK pathways, such as Akt and ERK1/2, respectively, are present in photoreceptor nuclei and are regulated by insulin and light.

1. Introduction

Phototransduction occurs in the outer segments of photoreceptor cells. Other signaling mechanisms, such as phosphoinositide cycle, phosphoinositide 3-kinase (PI3K) pathway and insulin receptor (IR) activation, have been reported to be activated by light in this portion of the photoreceptor cell (Ghalayini et al., 1998; Ghalayini and Anderson, 1995; Guo et al., 1997; Huang et al., 2000; Ilincheta de Boschero and Giusto, 1992; Rajala et al., 2007,

Abbreviations: DAGK, diacylglycerol kinase; ERK, extracellular signal-regulated kinase; p-ERK, phosphorylated extracellular signal-regulated kinase; p-Akt, phosphorylated Akt; p-p38, phosphorylated p38; LAP2-β, lamina-associated polypeptide 2 beta isoform; CRX, cone-rod-homeobox transcription factor; PI-PLC, phosphatidylinositol specific phospholipase C; IR, insulin receptor; pIR, phosphorylated insulin receptor; PNF, photoreceptor nuclear fraction; RNF, retinal nuclear fraction; DAG, diacylglycerol; PA, phosphatidic acid; PPI, polyphosphoinositides.

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2002). However, little is known about the possible consequences of light stimuli at the nuclear level of photoreceptor cells.

Recent findings from our laboratory have reported that light stimulation activates signal transduction pathways at the nuclear level of photoreceptor cells (Natalini et al., 2014). The exposure of retinas to light induces a partial depletion of two diacylglycerol kinase (DAGK) isoforms (DAGKε and β) and promotes the increase of DAGKζ in photoreceptor nuclei. A light-dependent phosphoinositide-specific phospholipase C (PI-PLC) activation has been found to be related to PA formation at the nuclear level. Further findings from our laboratory showed that light increases phosphorylated protein kinase C α (p-PKCα) in the nuclear fraction and suggested that this protein kinase could be modulated by DAGK activity in the nucleus (Natalini et al., 2014). The modulation of either nuclear PKCα or its possible regulator DAGK could be regulated by upstream light-activated photoreceptor components also present in the nuclear fraction.

Previous studies showed that light induces tyrosine phosphorylation of retinal IR and that this activation leads to the binding of PI3K to ROS membranes (Rajala et al., 2002) and the subsequent activation of Akt (Li et al., 2008; Rajala et al., 2010). They also demonstrated that photobleaching of rhodopsin regulates the phosphorylation state of IR, IGF-1R, and insulin-related receptor (IRR), all of which belong to the same receptor-tyrosine kinase (RTK) family.

It is also known that 3-Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop of a number of protein serine/threonine kinases of the AGC kinase superfamily, including Akt/PKB and PKC isoforms. Interestingly, it has been recently reported that phosphoinositide-dependent phosphorylation of PDK1 regulates its nuclear translocation (Scheid et al., 2005). Further research revealed that insulin-like growth factor (IGF)-I produces a transient elevation of intranuclear DAG levels and an increase in the content of PKC in the nucleus of Swiss 3T3 cells, intranuclear DAGK being involved in terminating PKC-mediated signaling events (Martelli et al., 2000).

Therefore, based on our findings showing light-dependent changes in nuclear DAGK and in p-PKC, we explored if these light-dependent changes are produced in relation to IR activation by light. In view of this, the aim of the present work was to explore the effects of light on insulin-related signaling pathways, such as mitogen-activated protein kinase (MAPK) and PI3K pathways, as well as the effects of insulin on DAGK activity and phosphoinositide phosphorylation in nuclei from photoreceptor cells. To this end, the presence of IR and pIR in photoreceptor nuclei was studied and their content in nuclei from retinas exposed to light or darkness was compared.

This work provides the first lines of evidence that confirm the presence of components of signaling pathways linked to G-protein coupled receptors and TRKs, such as Akt and ERK1/2, in photoreceptor nuclei and their regulation by light. Both Akt and ERK1/2 are activated in photoreceptor nuclei from retinas exposed to light with respect to those kept in darkness. Findings from the present study confirm the presence of IR in photoreceptor nuclei and show that its content is modulated by light and by the exposure of bovine retinas to insulin. In addition, our results demonstrate that light modulates IR activation. They also reveal that *in situ* insulin treatment induces a significant increase in DAGK and in phosphoinositide kinase activities in photoreceptor nuclei from retinas exposed to light.

2. Materials and methods

2.1. Materials

Bovine eyes were obtained from a local abattoir, placed on ice within 10 min of the animal's death, and subsequently kept in darkness during their transfer to the laboratory. Polyclonal antibody raised against CRX was generously supplied by Dr. C. Craft (University of Southern California, Los Angeles, USA). Mouse monoclonal anti-LAP-2β (#611000) from BD Biosciences (San Jose, CA, USA) was generously supplied by Dr. Ana Ves Losada (Instituto de Investigaciones Bioquímicas de La Plata, Argentina). Rabbit polyclonal antibodies anti-p-Akt (#9275), anti-Akt (#9272), anti-p-ERK1/2 (#9101), anti-ERK1/2 (#9102), and rabbit monoclonal and anti-insulin receptor β (#3025) were purchased from Cell Signalling (Beverly, MA, USA). Rabbit polyclonal anti-p-insulin receptor β (#44806G) was purchased from Life Technology. Anti-p-p38 (sc-101759) and polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and polyclonal HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 546 (goat anti-rabbit IgG) A11035, and TO-PRO®-3 Stain, from Life Technologies Corporation, were generously provided by Dr. Luis E. Politi (Instituto de Investigaciones Bioquímicas de Bahía Blanca, Argentina).

Supplies, such as U73122 and LY294002 from Sigma Aldrich Corporation, USA, were generously provided by Dr. N. Bazán (Neuroscience Center of Excellence at Louisiana State University Health Sciences Center, New Orleans, USA). Latrunculin was purchased from Molecular Probes Inc. (Eugene, OR). Radioactive substrates [γ-^{32}P]ATP (10 Ci/mmol) and Preblended Dry Fluor 2a70 for scintillation cocktail were obtained from Research Products International Corp, USA. Insulin and all the other chemicals used in the present research were purchased from Sigma Aldrich (St. Luis, MO, USA).

2.2. Light–darkness protocol applied to bovine retinas and isolated nuclear fractions. Isolation of retinal nuclear fraction (RNF) and photoreceptor nuclear fraction (PNF)

In order to analyze the effect of light on a photoreceptor cell nuclear population, bovine eyes were treated as previously described (Natalini et al., 2014). Briefly, after a 2.5 h darkness adaptation period, the cornea, lens and aqueous humor were removed under dim red light. Eyecups were placed on ice and filled with oxygenated (95% O_2, 5% CO_2) Ames medium (2 mg/ml glucose, 119.5 mM NaCl, 3.6 mM KCl, 0.1 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1.15 mM $CaCl_2$, 22.6 mM $NaHCO_3$, pH 7.33). Then, eyecups were either kept in darkness or exposed to light (288 cd/m^2) for 30 min. For nuclear fraction isolation retinas were dissected from the eyes after darkness or light exposure and homogenized (30% weight/vol) with 0.25 M sucrose TKM medium (50 mM Tris–HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ and 1 mM EGTA in the presence of protease inhibitors). To isolate a retinal nuclear fraction (RNF) the homogenate was filtered and two volumes of 2.3 M sucrose in TKM were added to reach a sucrose concentration of 1.6 M. The homogenate in 1.6 M sucrose solution (8.4 ml) was placed onto a 2.3 M sucrose layer (2.7 ml) and centrifuged at 130,000 *g* for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge. RNF was obtained in the pellet of the gradient (Natalini et al., 2014).

In order to purify a photoreceptor nuclear fraction (PNF) retinal homogenate (8.4 ml, 1.6 M sucrose) was placed onto a 2.2 M (1.4 ml) sucrose and 2.4 M (1.4 ml) sucrose TKM gradient and centrifuged at 106,000 *g* for 70 min using a Beckman SW41 rotor in a Beckman Optima LK-90 ultracentrifuge and PNF was obtained in the pellet of the gradient (Natalini et al., 2014). Both nuclear fractions were briefly washed with TKM buffer in order to eliminate the high density sucrose solution and ice-cold buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT) was placed in contact with the pellet for 30 min before resuspension. The

purity of PNF obtained with this protocol has been previously demonstrated through confocal microscopy (IF) and electron microscopy. Furthermore, the absence of calnexin (ER marker) and rhodopsin as well as the enrichment in CRX in PNF was demonstrated by WB assays (Natalini et al., 2014). Periodical controls were performed in order to assure the purity of the fraction.

2.3. Determination of DAGK activity

DAGK activity in PNF obtained from bovine retinas exposed to light or darkness was studied in PNF by measuring radiolabeled PA formation using [γ-^{32}P]ATP (3 μCi) and endogenous DAG. The enzyme reaction was carried out in a buffer containing 50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT in a volume of 200 μl using 100 μg of PNF proteins per assay. The reaction was carried out at 37 °C for 10 min and stopped by the addition of chloroform/methanol/1NHCl (2:1:0.2, by vol). Blanks were prepared identically except that membranes were boiled for 5 min before use. Lipids were extracted according to Folch (Folch et al., 1957) and five additional washes of the lipid extract were performed using theoretical upper phase (chloroform/methanol/water, 3:48:47 by vol) in order to eliminate [γ-^{32}P]ATP. Lipids were subsequently separated by one-dimensional thin-layer chromatography (TLC) using 1% potassium oxalate in silica gel H plates (Merck) in a mobile phase consisting of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol) (Ilincheta de Boschero and Giusto, 1992; Podlecki et al., 1987). The plates were exposed to iodine vapors, PA spots were identified using a PA standard and scraped off for counting by liquid scintillation spectroscopy. Protein content was determined following Lowry (Lowry et al., 1951).

2.3.1. Insulin eyecup exposure under darkness or light conditions

Before carrying out the light–darkness protocol, eyecups filled with oxigenated Ames medium with 0.5% glucose were divided into two groups: the control condition (without insulin plus 0.2 mM vanadate) and the experimental condition (with 0.2 μM insulin plus 0.2 mM vanadate). Vanadate, a tyrosine phosphatase inhibitor, was included in the assay in order to protect insulin-dependent protein tyrosine phosphorylation. Half of the eyecups from both groups was maintained under dim red light while the other half was exposed to light condition for 30 min (light–darkness protocol). PNF was subsequently obtained and resuspended as previously described (2.2.).

DAGK activity assay was performed using [γ-^{32}P]ATP in the absence of detergents and in the presence of endogenous DAG. Enzyme reaction, lipid isolation and quantification of radiolabeled PA were carried out as described above (2.3.1.).

2.3.2. Insulin exposure of isolated nuclei obtained under darkness or light conditions

After carrying out the light/darkness protocol, PNF was obtained and resuspended as previously described (2.2). DAGK activity assay was performed using [γ-^{32}P]ATP in the absence of detergents and in the presence of endogenous DAG. PNF was incubated for 10 min in the absence or in the presence of insulin (0.2 or 0.8 μM) plus 0.2 mM vanadate. Alternatively, the effect of insulin was also analyzed in PNF previously preincubated with 0.2 mM vanadate in order to protect endogenous protein tyrosine phosphorylation.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (WB) assays

PNF and RNF containing nuclear proteins were denatured with Laemmli sample buffer at 100 °C for 5 min (Laemmli, 1970) and 40 μg protein were resolved in a 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). PVDF membranes were blocked with 10% bovine seroalbumin (BSA) in TTBS buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl and 0.1% (weight/vol) Tween 20] at room temperature for 2 h. Membranes were subsequently incubated with primary antibodies at room temperature for 2 h:mouse monoclonal anti-LAP-2β (1:2000 vol:vol), rabbit polyclonal anti-CRX (1:2000, vol:vol), rabbit polyclonal anti-p-Akt (1:1000 vol:vol), anti-Akt (1:1000 vol:vol), anti-p-p38 (1:1000 vol:vol), anti-p-ERK1/2 (1:1000 vol:vol), anti-ERK1/2 (1:1000 vol:vol), anti-insulin receptor β (1:500 vol:vol) and anti-p-insulin receptor (1:500 vol:vol). After four washes in TTBS, membranes were exposed to the appropriate HRP-conjugated secondary antibody at room temperature for 2 h. Membranes were washed again three times with TTBS and immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences) using standard x-ray film (Kodak X-Omat AR). Densitometry values of the immunoreactive bands were determined using ImageJ 1.38 software (Schneider et al., 2012). The molecular weight of bands was determined using the spectra multicolor broad range protein ladder (Thermo Scientific).

2.5. Immunofluorescence (IF) in isolated photoreceptor nuclei from bovine retinas

1) To test both IR presence and localization at the nuclear level and the effect of the exposure of retinas to light, equal suspensions of PNF from either light-exposed retinas or darkness-adapted retinas were fixed with paraformaldehyde (2%) and after 1 h incubation samples were centrifuged at 4500 *g* for 20 min. 2) To test the potential involvement of microfilaments in light-dependent IR localization in PNF, latrunculin, which inhibits actin polimerization and disrupts microfilament mediated processes, was used. Bovine eyecups were incubated with 2.5 μM latrunculin or vehicle for 15 min under darkness condition and then were subsequently exposed to light or kept in darkness as usual. PNF were isolated and the samples were fixed with paraformaldehyde (2%) and after 1 h incubation samples were centrifuged at 4500 *g* for 20 min.

The pellets from both protocols were resuspended in buffer to finish the fixation period. A drop from each condition was subsequently placed on a slide and was left for 15–30 min to allow drying. Nuclei were permeabilized with 0.1% of Triton X-100 at room temperature for 15 min, washed three times at room temperature for 5 min with high ionic strength Hepes-buffered solution (BAFI) (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 2 mM EGTA and 1 mM DTT) and blocked with 5% BSA. Rabbit polyclonal anti-insulin receptor antibody (anti-IR) diluted in 5% BSA (1:50, vol:vol) was added and the mixture was incubated overnight at 4 °C. After three washes with BAFI, nuclei were incubated at room temperature with Alexa Fluor 546 (goat anti-rabbit IgG) A11035 (1/1000 vol:vol) for 2 h. After three further washes with BAFI, samples were incubated with TO-PRO-3® (1:1000 vol:vol) for 25 min. Primary antibody was omitted in the negative control and no nonspecific labeling was detected under this condition. The control of nuclei presence was carried out in the presence of TO-PRO-3 as fluorescent stain. Samples were imaged using a TCS-SP2 confocal microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) equipped with an acousto-optical beam splitter and a ×63 (1.2 numerical aperture) water immersion objective. Quantification analyses of fluorescent intensities were carried out using ImageJ 1.38 software (Abramoff et al., 2004; Rasband, 1997) and multiple selections of slides were analyzed using the Region of Interest (ROI) tool.

2.6. Statistical analysis

Statistical analysis was performed using either ANOVA followed by Bonferroni's test to compare means or student's t-test, both using GraphPad Prism software. p-Values lower than 0.05 were considered statistically significant (indicated with asterisks). Data represent the mean value ± SD of three independent experiments. The WB assays shown are representative of three analyses carried out in samples from three independent experiments.

3. Results

3.1. Light effects on insulin-related signaling pathways in PNF

Taking into account that light induces insulin receptor activation in the outer segment of photoreceptor cells (Rajala et al., 2002) and that our previous results demonstrate nuclear activation of different lipid-related enzymes in photoreceptor nuclei from bovine retinas exposed to light (Natalini et al., 2014), our first purpose was to study the effect of light on insulin-related signaling pathways in PNF. To this end, WB assays were carried out to determine total Akt and ERK1/2 levels as well as the phosphorylated forms of these kinases and p38 in PNF from darkness-adapted or from light-exposed bovine retinas as detailed in Materials and Methods. CRX, a specific transcription factor of photoreceptor cells (Furukawa et al., 1997), was determined as loading control.

The exposure of retinas to light induced a significant increase in the content of total ERK1/2 and p-ERK1/2 (2-fold) (Fig. 1a) and in the content of p-p38 (60%) (Fig. 1b) in PNF. No changes were observed in the p-ERK1/2/ERK1/2 ratio, indicating that light induces the translocation of p-ERK1/2 to the nucleus (Fig. 1a). In addition, an increase in the content of p-Akt (3-fold) without changes in total Akt was observed in nuclei from retinas exposed to light (Fig. 1c). These data reveal for the first time that exposure of retinas to light induces changes in the nuclear content of components that usually participate in insulin-elicited signaling pathways.

3.2. Insulin effects on DAGK activity in PNF from retinas exposed to light or darkness

It is known that bovine rod outer segments (ROS) contain p85 and p110 subunits of class 1 PI3K, a complex which is significantly active in light-adapted retinas *in vitro* (Guo et al., 1997) and which can be activated *in vivo* through light-induced tyrosine phosphorylation of IR in ROS (Rajala et al., 2002). Previous results from our laboratory demonstrated that insulin regulates synaptosomal DAGK through PI-PLC activation (Zulian et al., 2009, 2011). Based on our observations of a different localization of DAGK isoforms and of the changes in DAGK activity in response to light at the nuclear level (Natalini et al., 2014), we explored if insulin induces changes in DAGK activity in photoreceptor nuclei from bovine retinas exposed either to light or darkness.

DAGK activity was analyzed by measuring PA formation through [γ-^{32}P]ATP incorporation in PNF. To this end, prior to PNF isolation, retinas were preincubated for 15 min with 0.2 μM insulin plus 0.2 mM vanadate (I + V) in order to protect tyrosine phosphorylation induced by insulin-dependent IR activation, and they were subsequently exposed to light or darkness for 30 min as detailed in Materials and Methods. On the other hand, in order to analyze the direct effects of insulin on photoreceptor nuclei, the PNF fraction from darkness-adapted or light-exposed retinas was incubated with I + V as described above. A third condition was also analyzed consisting in insulin treatment of PNF (nuclear exposure) isolated from retinas exposed to insulin either under light or dark conditions. [γ-^{32}P]-PA formation was measured and results were expressed as mean of incorporation values expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$(Fig. 2).

Bovine retinas incubation with I + V significantly decreased PA formation in PNF from retinas exposed to light (26%) with respect to the control condition. In contrast, I + V induced no changes with respect to the control condition under dark condition.

Interestingly, when PNF fraction was directly incubated with I + V (without eyecup incubation with I + V), a significant increase in PA formation was observed both under light condition (18%) and dark condition (25%) (Fig. 2). In addition, when PNF obtained from retinas previously exposed to I + V in darkness was subsequently treated with I + V, a stimulation in nuclear PA formation (24%) could be observed, this increase being similar to that observed when PNF was treated with I + V (Fig. 2). In contrast, when PNF was obtained from retinas preincubated with I + V under light condition and subsequently incubated with I + V, no changes in PA formation were observed with respect to the control condition (Fig. 2). These results suggest that under light condition insulin exerts two differential effects, inhibiting nuclear DAGK activity when the eyecup is exposed to I + V but stimulating this kinase when PNF is directly incubated with I + V. Furthermore, the results obtained under the third condition (retina and PNF exposure) suggest that these two differential effects of insulin are compensated. Our results demonstrate that insulin regulates nuclear DAGK activity locally and also through signaling transduction pathway activated in entire retinas.

Previous WB results from our laboratory confirmed a light-dependent nuclear enrichment in DAGKζ content and depletion in DAGKε and DAGKβ content (Natalini et al., 2014). The possibility that insulin in the eyecup regulates the light-dependent content of DAGK isoforms in PNF cannot be ruled out. This could explain the insulin-mediated effects observed on PA formation in PNF from light-exposed retinas.

3.3. Direct insulin effects on nuclear DAGK activity. Modulation by PIP_2-PLC and PI3K

Taking into account the light-dependent differential location of DAGK isoforms in photoreceptor nuclei (Natalini et al., 2014) and the modulation of DAGK activity by insulin (Fig. 2), we further explored in PNF from dark adapted retinas (to avoid changes in DAGKs localization induced by light) insulin-dependent activation of two insulin-elicited signaling pathways in synaptic endings and in photoreceptor cells, PI-PLC (Zulian et al., 2009) and PI3K (Rajala et al., 2007, 2002, 2003), respectively. To this end, PNF was preincubated with 10 μM U73122 (PI-PLC inhibitor) and 10 μM LY294002 (PI3K inhibitor) in the presence of 0.2 mM vanadate and 20 mM NaF (as Ser-Threo phosphatases inhibitor) prior to insulin treatment.

As shown in Fig. 3a, nuclear PA formation was observed to be strongly increased by insulin. This increased PA formation induced by insulin was significantly inhibited when U73122 was present in the preincubation period. However, no significant inhibition was observed when PNF was preincubated with LY294002. These results suggest that PI-PLC modulates insulin-elicited PA formation in the nucleus of photoreceptor cells.

Under these experimental conditions, phosphorylation of nuclear polyphosphoinositides (PPIs) was also measured through the incorporation of [γ-^{32}P]ATP in phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP2) and phosphatidylinositol triphosphate (PIP3) isolated by TLC chromatography isolation procedure. Insulin plus vanadate incubation significantly increased the phosphorylation of PPIs (Fig. 3b). Preincubation of PNF with LY294002 prior to insulin exposure significantly reduced

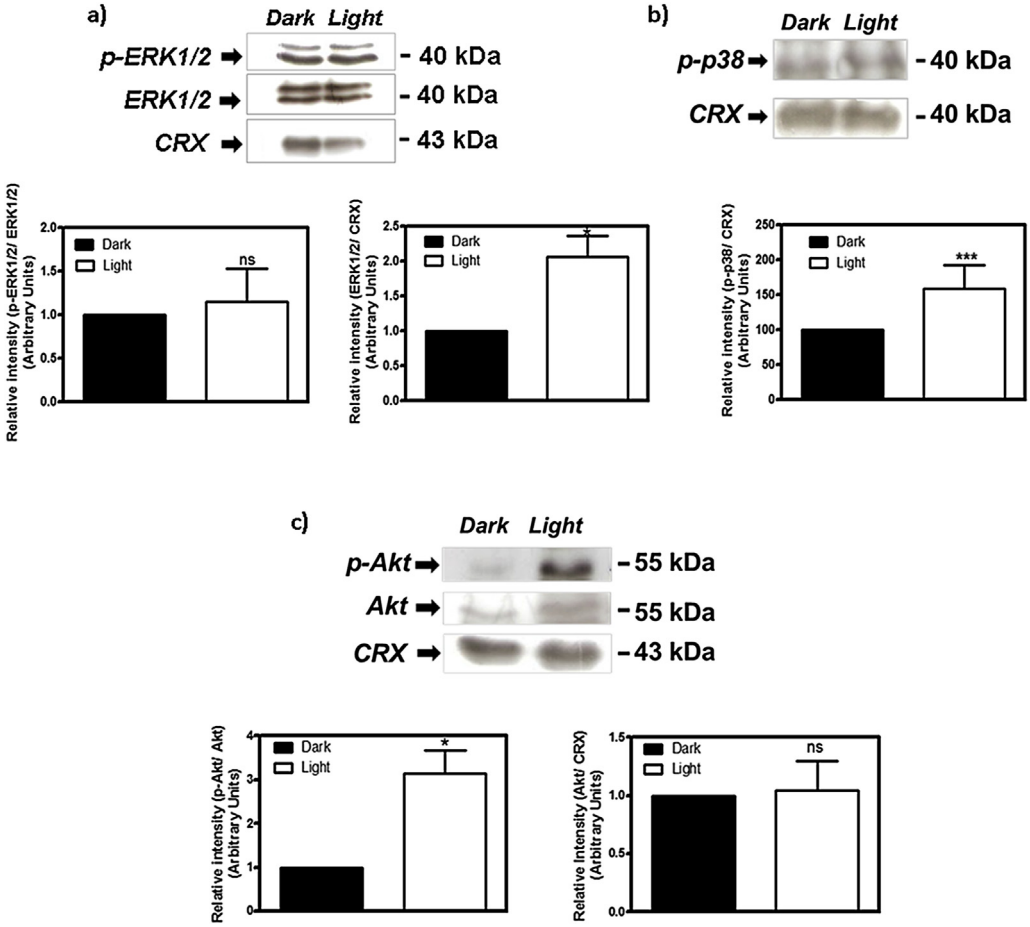

Fig. 1. Effect of light on the localization and activation of ERK1/2, Akt and p38 in PNF. WBs assays were performed to detect ERK1/2 and Akt total level as well as the phosphorylated forms (Figure a and c) and p-p38 content (Figure b) in PNF from light or darkness exposed retinas. PNF proteins (30 μg) were resolved in a 10% SDS-PAGE and transferred to a PDVF membrane. Membranes were blocked and incubated with primary and secondary antibodies as detailed in Materials and Methods. Immunoreactive bands were detected by enhanced chemiluminescence. One representative image of three independent WB is shown and numbers to the right indicate molecular weights. The bar graph shows the densitometry values of each phosphorylate form/total form, or the total form/CRX, expressed as ratio of the control (Dark condition). Error bars indicate standard error. Statistical analysis was performed using one way ANOVA followed by Bonferroni's test to compare means using GraphPad Prism software. Asterisks indicate significant differences with respect to the control (*$p < 0.05$, ***$p < 0.001$).

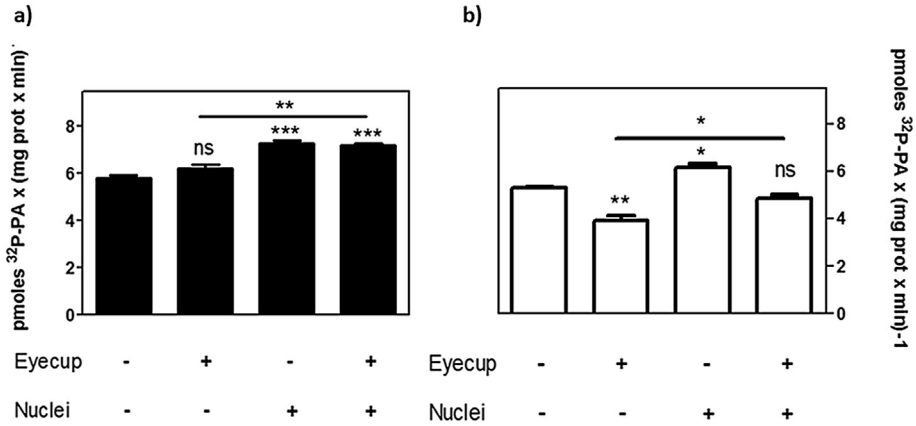

Fig. 2. Insulin effect on DAGK activity in PNF. Bovine eyecups were preincubated with 0.2 μM insulin and 0.2 mM vanadate, or the vehicles (control condition) in oxygenated Ames medium for 15 min under red dim light. Then, the eyecups were exposed to light or kept in darkness for 30 min, and the PNF were isolated as was described in Material and Methods. The isolated PNFs were then incubated with 0.2 μM insulin or its vehicle and 0.2 mM vanadate and DAGK activity was measured using [γ-^{32}P]ATP (3 μCi per assay), 250 μM ATP and endogenous DAG as reaction substrate, for 10 min at 37 °C. Data (means of incorporation values) are expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$. Error bars indicate standard error. Statistical analysis was performed using one way ANOVA followed by Bonferroni's test to compare means using GraphPad Prism software. Asterisks over error bars indicate significant differences between the experimental conditions and the control condition (***$p < 0.001$, **$p < 0.01$). Asterisks over a line indicate comparison between "eyecup+; nuclei−" and "eyecup+; nuclei+".

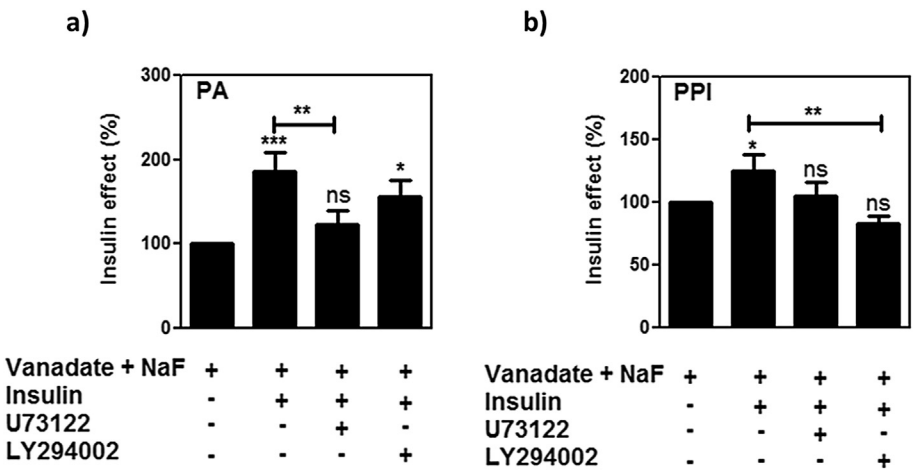

Fig. 3. Direct insulin effect on nuclear DAGK activity. Modulation by PIP_2-PLC and PI3K. PNF from bovine retinas kept in darkness were preincubated for 10 min with 0.2 mM vanadate, 20 mM. NaF and the PI-PLC inhibitor U73122 (10 μM) or the PI3K inhibitor LY294002 (10 μM), or with vanadate and NaF plus the vehicle of the inhibitors (control condition). Then, PNF was incubated with 0.2 μM insulin, or with the insulin vehicle, and DAGK activity was measured as described in Fig. 2. PA (figure a) and PI (figure b) levels were quantified as described in Materials and Methods. Data are expressed as insulin effect (%), considering PA or PPI formation in the control condition as 100%. Error bars indicate standard error. Statistical analysis was performed using one way ANOVA followed by Bonferroni's test to compare means using GraphPad Prism software. Asterisks indicate significant differences between the experimental conditions and the control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). The asterisks over the horizontal lines indicate significant differences between different experimental conditions.

the effect of insulin on PPIs phosphorylation. In contrast, the presence of U73122 during the preincubation period did not reverse the stimulatory effect of insulin on the phosphorylation of PPIs (Fig. 3b).

3.4. Direct insulin effects on DAGK activity in PNF obtained from retinas exposed to light or dark conditions

Fig. 2 shows insulin's (0.2 μM) direct effects on nuclear DAGK activity when PNF was obtained from retinas exposed to light or kept in darkness. Previous research confirmed that IR phosphorylation and PI3K activation occur in retinal organ cultures exposed to 1 μM insulin (Li et al., 2008). We thus planned to study the effect of higher insulin concentrations on DAGK activity in PNF. To this end, DAGK activity was measured through PA synthesis by using [γ-^{32}P] ATP as radioactive precursor in the PNF from retinas exposed to light or darkness, preincubated with 0.2 mM vanadate for 10 min, and subsequently incubated in the presence or absence of 0.8 μM insulin.

A significant increase in PA formation was observed when PNF from bovine retinas exposed to light were incubated with 0.8 μM insulin (65%) with respect to the condition without insulin (Fig. 4a). Still, insulin incubation was observed to induce no significant changes in PA formation in PNF from retinas kept in darkness with respect to the condition without insulin (Fig. 4a). These results suggest that the effects of insulin on DAGK activity in PNF are mediated by light.

Taking into account that in previous experiments 0.2 μM insulin and vanadate (I + V) had been added simultaneously (Fig. 2), 0.8 μM insulin plus 0.2 mM vanadate were then added simultaneously in order to compare the effect of both insulin concentrations (Fig. 4b). It could be observed that 0.8 μM insulin induced a higher increase in DAGK activity in PNF from light and darkness conditions than 0.2 μM insulin (92% and 45% with respect to the control without insulin, respectively). Furthermore, in PNF treated with 0.8 μM insulin, significant differences in PA formation between light and darkness conditions were detected (Fig. 4b).

It is worthy of note that when PNF obtained from retinas kept in darkness were preincubated with vanadate (prior to 0.8 μM insulin exposure), no significant increase in DAGK activity induced by insulin was observed with respect to the control condition (Fig. 4a). It could thus be hypothesized that in the absence of insulin a basal tyrosine phosphorylated state in PNF (protected by vanadate) increases DAGK activation, whereas in PNF preincubated with vanadate, insulin does not induce PA formation. In contrast, in the absence of vanadate preincubation insulin-dependent DAGK activation could be observed (Fig. 4b). To confirm this hypothesis, a comparative experiment with and without vanadate preincubation was carried out.

As shown in Fig. 5a, in PNF from dark-exposed retinas, vanadate preincubation of nuclei was found to significantly increase DAGK activity (59%) whereas 0.8 μM insulin exposure was observed to slightly increase PA formation (20%) with respect to the control with vanadate. In contrast, in PNF from light-exposed retinas (Fig. 5b), 0.8 μM insulin was found to significantly increase DAGK activity (68%) with respect to the control with vanadate. Taken together, these findings suggest that although an activated IR is present in PNF from retinas kept in darkness, light may regulate its content.

3.5. Insulin receptor presence and phosphorylation in PNF from bovine retinas exposed to light or darkness

In order to determine the presence of IR in photoreceptor nuclei from retinas exposed to light or darkness, WB assays were carried out using an anti-IR β antibody and an anti-CRX as loading control. WBs showed that IR is present in PNF from both conditions, its content being 28% higher in PNF from retinas exposed to light than in PNF from retinas exposed to darkness (Fig. 6a). IR was also observed in RNF which includes nuclei from all retina cell types. Fig. 6b shows the presence of IR in both nuclear fractions, its content being 91.5% higher in RNF than in PNF. This demonstrates that IR is not only present in photoreceptor nuclei but also in other retina cell nuclei. Furthermore, in order to analyze the IR activation state (receptor phosphorylation) in PNF isolated from retinas either exposed to light or maintained in darkness, WB assays were carried out using an anti-phospho-IR (Tyr(P)1158/Tyr(P)1162/Tyr(P)1163) antibody and anti-IR β as well as anti-LAP2-β as loading control.

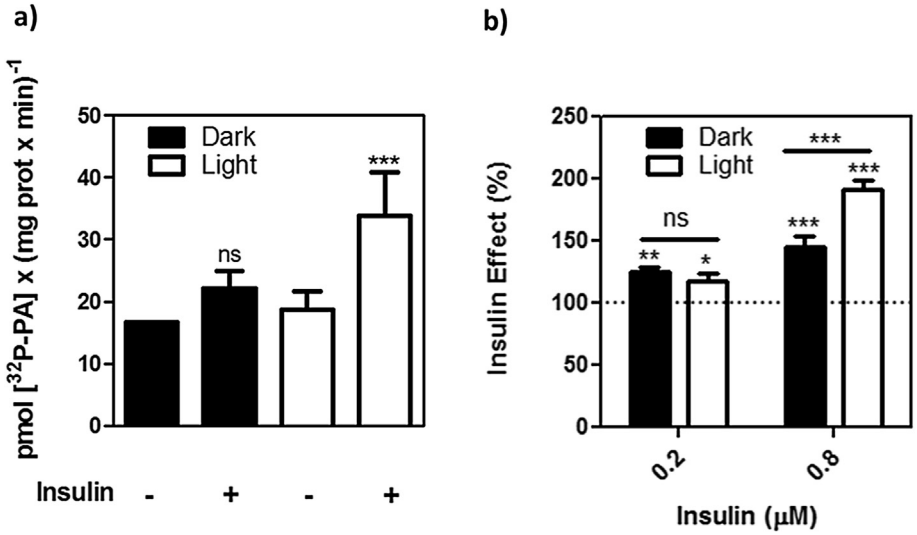

Fig. 4. Direct insulin effect on DAGK activity in PNF obtained from retinas exposed to light or to darkness. a) PNF from retinas exposed to light or kept in darkness were preincubated with 0.2 mM vanadate for 10 min and then incubated with 0.8 μM insulin or insulin vehicle. DAGK activity assay was performed as described in Fig. 2. Results are expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$. Data (means of incorporation values) are expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$. b) PNF from retinas exposed to light or kept in darkness were incubated with two different insulin concentrations (0.2 μM or 0.8 μM) or the vehicle of insulin (control conditions) plus 0.2 mM vanadate and DAGK assay was performed as described in Fig. 2. Data are expressed as insulin effect (%), considering each control condition (dark or light without insulin) as 100%. Statistical analysis in a) and b) was performed using one way ANOVA followed by Bonferroni's test to compare means using GraphPad Prism software. Asterisks indicate significant differences between the experimental conditions and the control conditions. The asterisks over the horizontal lines indicate significant differences between experimental conditions (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

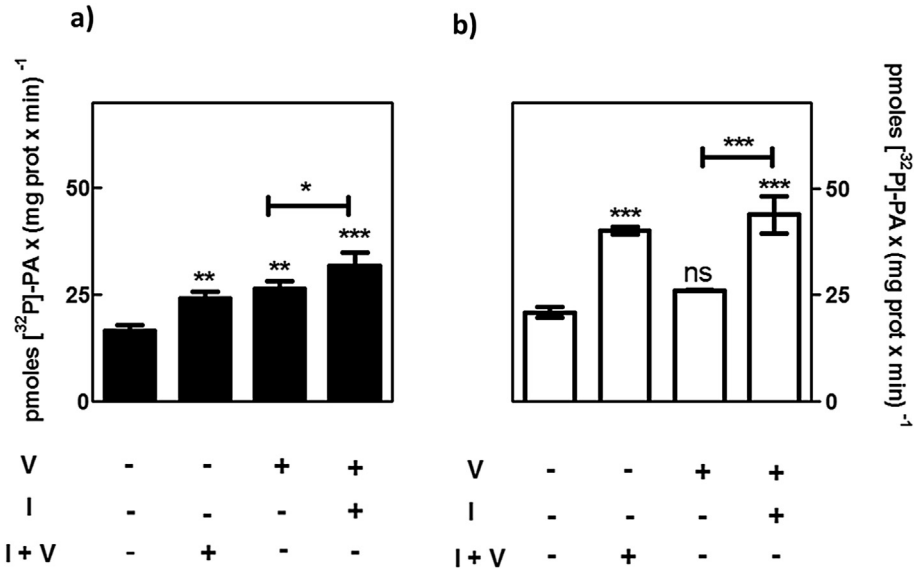

Fig. 5. Effect of vanadate preincubation on insulin-induced DAGK activity in PNF obtained from retinas exposed to light or darkness. PNF from retinas exposed to light (b) or kept in darkness (a) were preincubated with 0.2 mM vanadate (V) for 10 min and then incubated with 0.8 μM insulin (I), or were co-incubated with insulin plus vanadate (I + V). The vehicles of insulin and vanadate were included in the control condition. DAGK activity assay was performed as described in Fig. 2. Results are expressed as pmol [^{32}P-PA] × (mg prot × min)$^{-1}$. Asterisks indicate significant differences between the experimental conditions and the respective control conditions. Error bars indicate standard error. Statistical analysis was performed using one way ANOVA followed by Bonferroni's test to compare means using GraphPad Prism software. Asterisks over the horizontal lines indicate significant differences between these experimental conditions (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Results from this assay (Fig. 6c) showed that the exposure of retinas to light induced an increased ratio pIR/LAP2-β (approximately three fold) with respect to that found under the dark condition. In addition, no changes in the ratio pIR/IR were observed in PNF from retinas either exposed to light or maintained in darkness. These results suggested that IR detected in PNF is phosphorylated.

To further confirm the localization of IR in nuclei from photoreceptor cells, IF assays were carried out using anti-IR β antibody and TO-PRO3 as nuclear marker. They demonstrated the presence of intranuclearly distributed IR in photoreceptor nuclei from bovine retinas (Fig. 7). In addition, IR fluorescence intensity revealed that the content of IR was significantly higher in nuclei from bovine retinas exposed to light (25%) than in those under the control darkness condition (Fig. 7).

To test the potential involvement of microfilaments in the light-dependent IR localization in PNF, latrunculin, was used. After

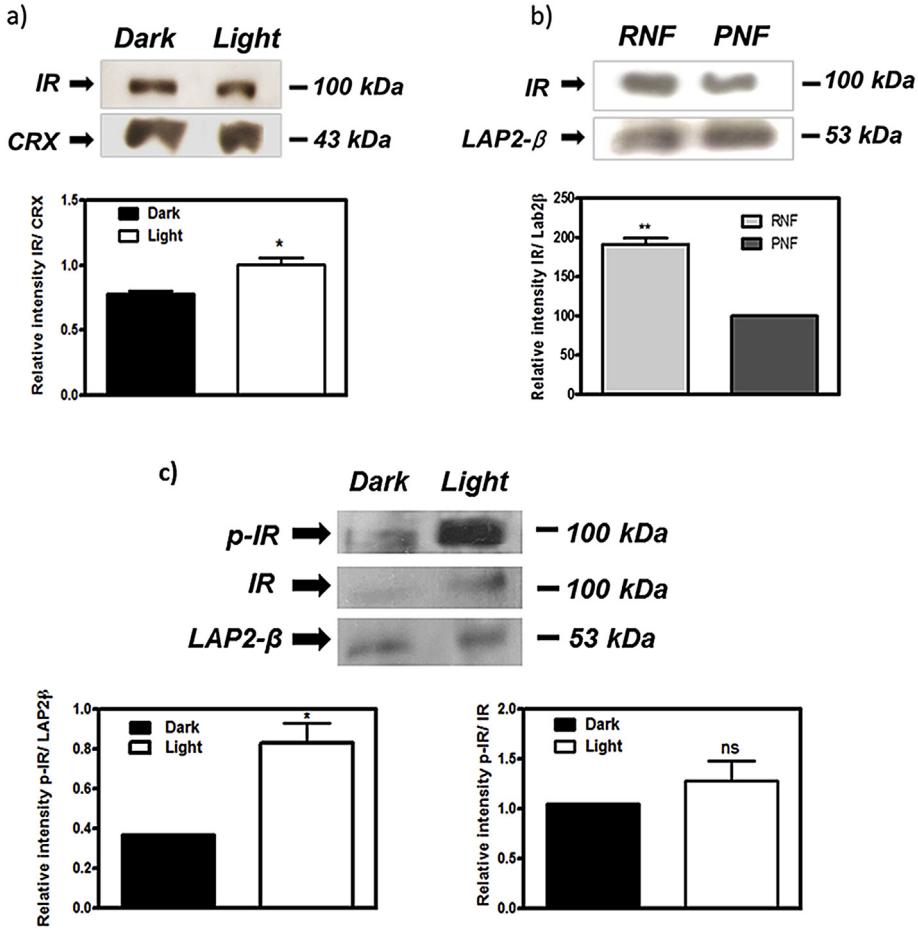

Fig. 6. IR presence and phosphorylation in PNF from bovine retinas exposed to light or to darkness. WBs assays were performed to detect IR and pIR content in PNF. a) WB assays were performed in PNF proteins from bovine retinas exposed to light or kept in darkness as described in Fig. 1. The bar graph shows the densitometry values of relative intensity of IR/CRX. b) IR content was determined by WB in PNF and RNF from light-exposed retinas as described in Fig. 1. The bar graph shows the densitometry values of IR/LAP2-β. c) pIR content was determined by WB in PNF from light-exposed or dark adapted retinas as described in Fig. 1. The bar graph shows the densitometry values of pIR/LAP2-β and pIR/IR. One representative image of three independent WB is shown and numbers to the right indicate molecular weights. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$).

15 min of incubation of retinas (eyecup exposure) with 2.5 μM latrunculin under dark condition, they were exposed to light as usual. Results from these experiments are shown as Supplementary Material (Fig. 1S). Latrunculin incubation clearly inhibited nuclear IR localization under light condition. IR fluorescence intensity from images revealed that the content of IR was significantly inhibited (25%) when bovine retinas were preincubated with latrunculin and subsequently exposed to light.

3.6. Insulin receptor presence in PNF from bovine retinas exposed to light and insulin

WB assays were also performed to detect IR in PNF obtained from eyecups incubated for 15 min under darkness condition with 0.8 μM insulin and 0.2 mM vanadate, and subsequently exposed to light for 30 min (Fig. 8a). As a control condition, WB assays were also performed in PNF from retinas exposed to light for 30 min and the isolated nuclear fraction was preincubated with vanadate prior to 0.8 μM insulin treatment (Fig. 8b). As shown in Fig. 8a, preincubation of retinas with insulin plus vanadate prior to light exposure increased IR content in PNF by 160% with respect to nuclei from retinas preincubated with the vehicle of insulin. As expected, no significant changes in IR content were observed when isolated PNF from light-exposed retinas was treated with insulin plus vanadate (Fig. 8b).

3.7. Direct insulin effects on ERK1/2 MAPK pathway in PNF from retinas exposed to light

As shown in Fig. 1a, the exposure of retinas to light induces pERK1/2 translocation to the nucleus of photoreceptor cells. Furthermore, Akt presence in nuclei, the increased content of nuclear p-Akt, and the increased content of p-p38 under light condition could be related to light-dependent IR activation. In addition, we also demonstrated 1) that IR is present in PNF, 2) that light and insulin induce an increased content of IR in PNF, 3) that exposure of retinas to light induces an increase in pIR content, 4) that DAGK activity is modulated by insulin in PNF (Figs. 3–5).

Taking into account that retinal ERK can be activated by insulin (Reiter et al., 2003), we explored if the presence and phosphorylation of ERK1/2 could be also regulated by insulin in PNF. To this end, total ERK1/2 and pERK1/2 were detected by WB in PNF preincubated with 0.2 mM vanadate and subsequently treated with or without 0.8 μM insulin. As can be seen in Fig. 9a, the ratio pERK1/2/ERK1/2 was increased by 200% when PNF was treated with 0.8 μM insulin. This demonstrates that IR is present in the nucleus of photoreceptor cells and that it is functional and can be activated by insulin.

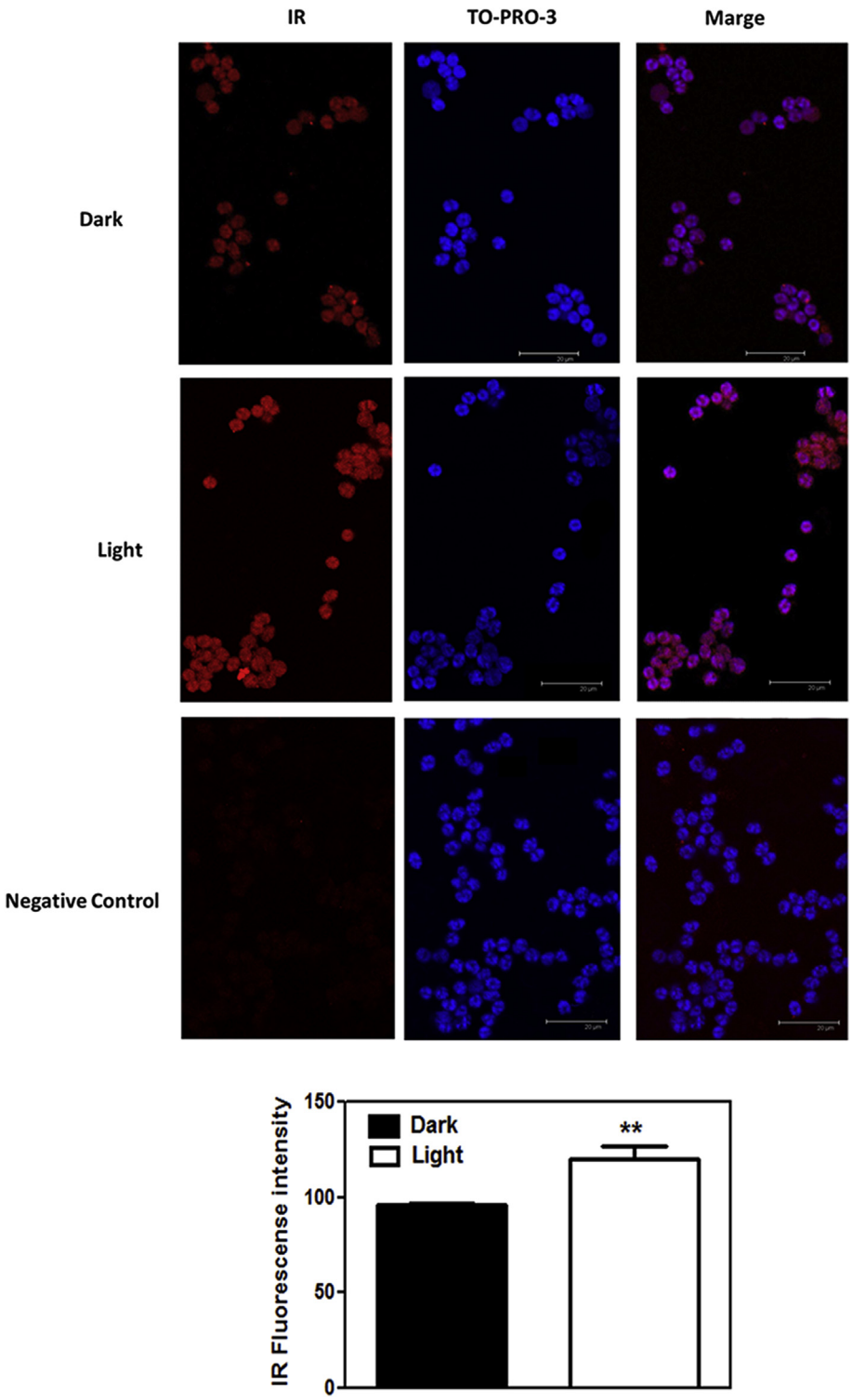

Fig. 7. Detection of IR in PNF from bovine retinas exposed to light or darkness by IF. PNF suspensions from bovine retinas exposed to light or kept in darkness were fixed, permeabilized and blocked with BSA as described in Materials and Methods. Then nuclei were incubated with the primary anti-IR antibody and the secondary antibody coupled to Alexa Fluor 546. TO-PRO-3 was used as nuclear marker as described in Materials and Methods. A negative control was performed in the absence of the anti-IR antibody. Samples were imaged with a confocal microscope. Representative images from three different experiments are shown. The bar graph shows IR fluorescence intensity. Asterisks indicate significant differences between RNF and PNF (**$p < 0.01$). Asterisks indicate significant differences with between the light and the dark control condition (control condition was considered as 100%) (**$p < 0.01$).

4. Discussion

The present work demonstrates that bovine retina's exposure to light induces changes in the content and activation of molecular components of the insulin signaling pathway in the nucleus of photoreceptor cells. It also shows that PNF from light-exposed retinas evidence higher levels of p-Akt, p-ERK1/2 and p-p38 than those from retinas kept in darkness (Fig. 1a, b and c). It is known

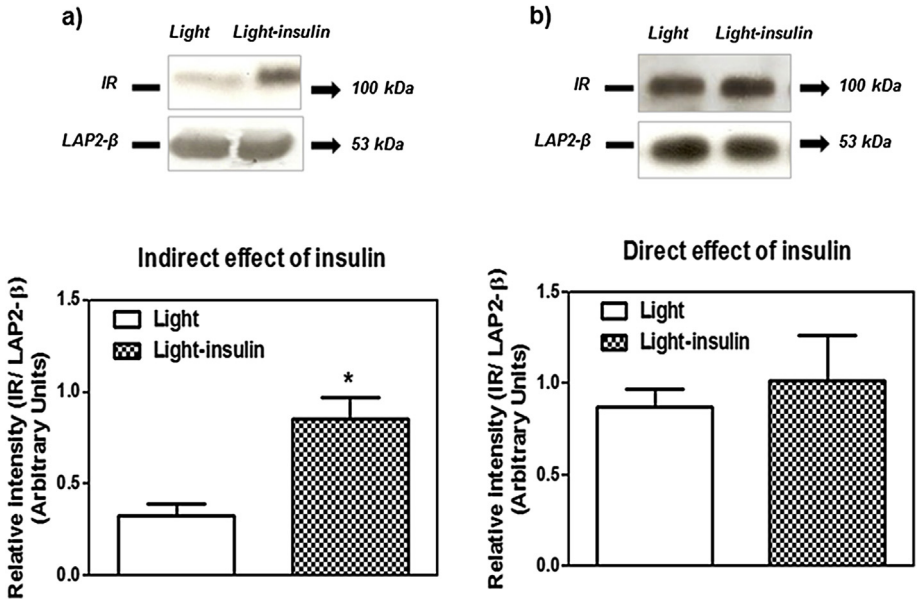

Fig. 8. IR presence in PNF from bovine retinas exposed to light and insulin. a) Bovine eyecups were preincubated with 0.8 μM insulin and 0.2 mM vanadate, or the vehicles (control condition) in Ames medium oxygenate (95% O_2, 5% CO_2) for 15 min under red dim light. Then, eyecups were exposed to light for 30 min, and the PNF were isolated as was described in Materials and Methods. IR was detected by WB as described in Fig. 1. b) Eyecups were exposed to light for 30 min, and PNF was isolated as was described in Materials and Methods. The PNF was preincubated with 0.2 mM vanadate for 10 min and then incubated with 0.8 μM insulin (or insulin vehicle) for 10 min at 37 °C. IR was detected by WB as described in Fig. 1. For a) b) one representative image of three independent WB is shown and numbers to the right indicate molecular weights. The bar graph shows the densitometry values of the relative intensity of IR/LAP2-β. Asterisks indicate significant differences between the experimental conditions (*$p < 0.05$).

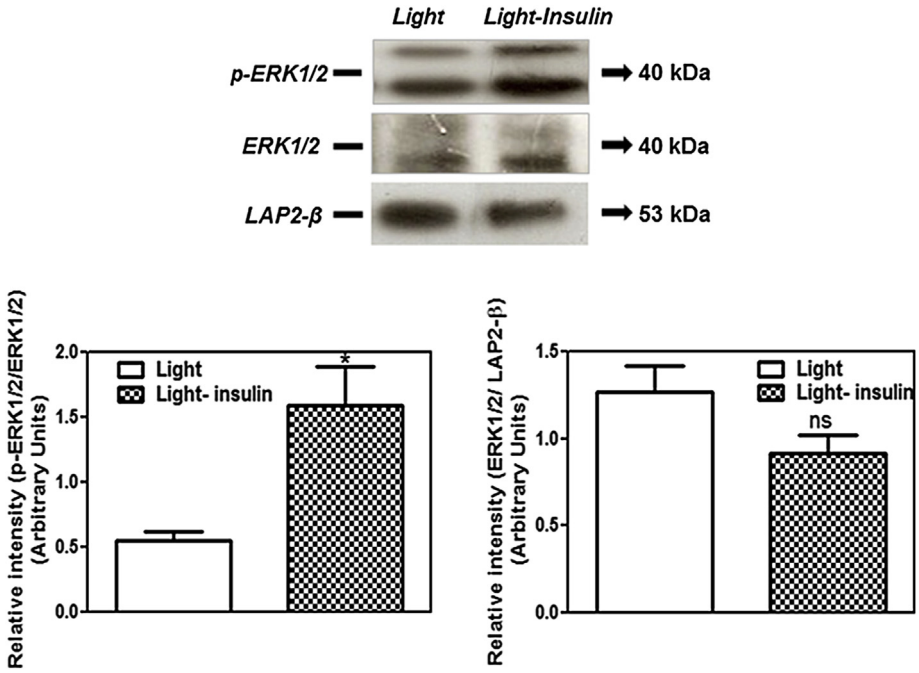

Fig. 9. Direct insulin effect on ERK1/2 phosphorylation in PNF from light-exposed retinas. Bovine eyecups were preincubated with 0.8 μM insulin and 0.2 mM vanadate, or the vehicles (control condition) in Ames medium oxygenate (95% O_2, 5% CO_2) for 15 min under red dim light. Then, eyecups were exposed to light for 30 min, and PNF was isolated as described in Material and Methods. WB assays were performed as described in Fig. 1 to detect pERK1/2, ERK1/2 and LAP2-β. One representative image of three independent WB is shown and numbers to the right indicate molecular weights. The bar graph shows the densitometry values of the relative intensity of pERK1/2/ERK1/2 and ERK1/2/LAP2-β. Asterisks indicate significant differences between the experimental conditions (*$p < 0.05$).

that IR is present in the retina (Reiter et al., 2003). Previous research has revealed that retina expresses several proteins associated to insulin-elicited signaling pathways. Further research confirmed the presence of IRS-1 and PI3K in rat retina (Folli et al., 1996). Gosbell and collaborators extended these observations to rat retina by demonstrating IRS-1 expression in ROS(Gosbell et al., 2000). PI3K light-dependent activation in ROS (Guo et al., 1997) and light-induced tyrosine phosphorylation of retinal IR were also reported (Rajala et al., 2002). It was also observed that IR activation leads to PI3K binding to ROS membranes (Rajala et al., 2002) and to the subsequent activation of Akt (Li et al., 2008; Rajala et al., 2010). Further research also demonstrated that IR activation is mediated

by the photobleaching of rhodopsin, a G-protein-coupled receptor, rather than by the phototransduction cascade (Rajala et al., 2007).

We have recently found that the exposure of bovine retinas to light induces relevant changes in the content of DAGK isoforms and phosphorylation of PKCα/β in the nuclei of photoreceptor cells (Natalini et al., 2014). In line with these findings, our present work reports for the first time that retina's exposure to light also induces important changes in molecular components of insulin signaling pathways in the nuclei of photoreceptor cells (Akt and ERK1/2 phosphorylation).

Taking into account that IR in the retina can be activated by light, we hypothesized that the effect of light on these insulin-related signaling pathways in PNF could be mediated by the activation of a photoreceptor IR. Previous research from our laboratory showed that insulin modulates DAGK activity in synaptic endings from rat cerebral cortex and hippocampus (Zulian et al., 2009). Further research from our laboratory confirmed that insulin stimulates DAGKε activity and the increased formation of stearoyl-arachidonoyl PA was suggested to be related to phosphoinositide synthesis. This stimulatory effect of insulin was observed to be mediated by PI-PLC activation (Zulian et al., 2011). Based on these findings, we explored if insulin either exogenously added to the retina (eyecup incubation) or added to isolated PNF could modulate nuclear DAGK activity.

Our experiments in which eyecups exposed to light were incubated with insulin (to analyze the effect of insulin on the retina) revealed an indirect inhibition of nuclear DAGK activity by insulin. In contrast, incubation of eyecups kept in darkness with insulin showed no significant changes in nuclear DAGK activity. These light-dependent effects of insulin on DAGK activity could be due to changes in the localization of DAGK isoforms in the nucleus of photoreceptor cells (Natalini et al., 2014). Whereas DAGKε and β nuclear localization was reduced, DAGKζ was increased in PNF from light-exposed retinas with respect to the darkness condition. The possibility that insulin in the eyecup regulates the light-dependent content of DAGK isoforms in PNF should not be ruled out.

In addition, a light-dependent extranuclear PI-PLC activation was found to be determinant for nuclear DAGK activity. Our studies suggested that DAG production as well as PIP_2 depletion induced by phospholipase activity regulate nuclear DAGK activity (Natalini et al., 2014). In view of these significant changes induced by light, further studies are necessary to fully understand the effects of insulin on nuclear DAGK activity in retinas exposed to insulin under light condition.

In addition, experiments carried out with PNF (to analyze insulin action in isolated nuclei) showed a direct modulation of nuclear DAGK activity by insulin. As shown in Fig. 2, PNF exposure to insulin stimulates PA formation in nuclei from darkness condition and light-exposed retinas.

It is interesting to note that the inhibition of nuclear DAGK activity induced by insulin (observed when PNF was obtained from retinas initially exposed to insulin under light condition) was reversed when PNF was directly exposed to insulin. Insulin thus seems to exert two different effects on nuclear DAGK activity under light conditions, namely an indirect inhibitory effect when the whole retina is exposed to insulin and a direct stimulatory effect when isolated nuclei are incubated with the hormone.

Furthermore, the direct effect of insulin on nuclear DAGK activity under the darkness condition seems to be mediated by insulin-dependent PI-PLC activation (Fig. 3). In contrast, increased phosphorylation of PIPs induced by PNF exposure to I + V was observed to be significantly inhibited by PI3K inhibitor LY294002, thus suggesting insulin-dependent PI3K activation in the nucleus.

IGF-I-elicited nuclear signaling events in culture cells were previously reported (Martelli et al., 2000; Neri et al., 1994, 1998). An inverse relationship in the nucleus of Swiss 3T3 cells between the levels of DAG and DAG kinase activity and transient PKCα translocation to the nucleus when these cells are stimulated by IGF-1 was reported (Martelli et al., 2000). Nuclear PI-PLC activation was suggested to be responsible for the translocation of PKCα to the nucleus (Neri et al., 1998). This could be indicative of a mediation mechanism of IGF-1 effects in cell proliferation. Still, in our model, insulin-dependent PI-PLC and DAGK activation at nuclear level could be related to a potential role of insulin in photoreceptor cell protection.

On the other hand, the insulin stimulatory effect on nuclear DAGK activity was also concentration-dependent. Whereas 0.2 μM insulin increased similarly PA formation in PNF obtained from light-exposed or darkness-maintained retinas (by 0.2 times with respect to their respective controls), 0.8 μM insulin under darkness condition increased PA formation in the nucleus by 1.5 times with respect to the control and, under light condition, insulin increased by two-fold PA formation with respect to the control condition (Fig. 4b).

Taken together, these findings demonstrate that the IR present in the nucleus of photoreceptor cells respond to insulin and that its activation leads to nuclear DAGK activation. The enhanced effects of insulin on DAGK activity observed under light conditions (Fig. 4b) were consistent with the increased nuclear IR content induced by light as shown by WB and IF studies (Figs. 6 and 7).

WB assays confirmed nuclear IR presence not only in nuclei from photoreceptor cells (PNF) but also in nuclei from other retinal cells (RNF) (Fig. 6). IR is a RTK and translocation to the nucleus seems to be a common feature for this class of receptors. A number of RTKs have been found in the nucleus, including receptors for growth hormone, several cytokines, epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) (Gomes et al., 2008; Lin et al., 2001; Reilly and Maher, 2001).

In several human cell lines showing high membrane IGF-1R expression, IGF-I exposure induces the internalization and degradation of the receptor and also nuclear translocation 15–60 min after addition of IGF-1 (Aleksic et al., 2010). Phosphorylated EGF receptor can be found in the nucleus within 1–2 min after stimulation with EGF, reaching highest levels within 15 min (Lin et al., 2001). Furthermore, phosphorylated hepatocyte growth factor receptor (HGFR) appears in the nucleus after stimulation with its agonist and this translocation has been linked to intranuclear formation of inositol trisphosphate (IP3) and initiation of Ca^{2+} signals within the nucleus (Gomes et al., 2008).

Studies carried out on cell lines have demonstrated that RTKs can translocate to the nucleus in cell lines and this translocation has also been observed to occur in primary hepatocytes. Autoradiographic studies on hepatocytes and hepatocyte lysates using a photosensitive insulin derivative (Podlecki et al., 1987) demonstrated that IR can be internalized and translocated to the nucleus of intact hepatocytes in a time- and temperature-dependent manner and also suggested that the nuclear translocation of the cell surface IR could be mediated by insulin's long-term effects.

Findings from the present study also provide the first lines of evidence of IR presence in the nucleus of bovine photoreceptor cells and show a light-dependent IR enrichment in the nucleus although IR is also present in PNF from retinas kept in darkness. In addition, the activation of IR in PNF from retinas exposed to light indicates that translocation to nuclei could be dependent on light activation (phosphorylation) of IR. Therefore, taking into account that in several RTKs a ligand-based activation condition induces translocation, it was hypothesized that light-dependent activation is also responsible for an pIR increase at nuclear level by means of this mechanism.

Although further experimental efforts are necessary, our recent

findings (Fig. 1S, Supplementary Material) indicate that actin network participates, at least partially, in the nuclear light-induced IR enrichment in bovine retina. In addition, it could not be discarded that in bovine retina IR enhanced nuclear content after insulin and/or light exposure occurs via the regulation of endocytic pathways or receptor recycling.

Our results also demonstrate that nuclear PA formation by DAGK activity is modulated by nuclear RTK on account of the fact that, in the absence of insulin, vanadate preincubation of PNF (which preserves RTK tyrosine phosphorylated state) significantly stimulates PA formation (Fig. 5). In addition, 0.8 μM insulin was observed to greatly enhance DAGK activity in PNF from retinas exposed to light with respect to the control darkness condition. A strong correlation between light-dependent increased IR presence and increased DAGK activity in PNF was also observed (Fig. 4). This confirms a positive light modulation of nuclear IR content which is in agreement with the light-dependent activation of insulin-activated signaling pathways in the nucleus, such as p-Akt, p-p38 and p-ERK1/2 (Fig. 1). In line with this, 0.8 μM insulin exposure of PNF from light-exposed retinas increased ERK1/2 phosphorylation (Fig. 9). In addition, although light-dependent pIR movilization would to be related to the neuroprotective role assigned to insulin at retinal level, no experimental evidence in support of this hypothesis was obtained in the present study.

Taken together, our findings suggest that the light-dependent increase in nuclear IR content as well as in nuclear phosphorylated components of PI3-K and ERK1/2 MAPK pathways is closely related to a light-dependent IR activation. In line with this, the regulation of nuclear DAGK activity by insulin reveals a novel light-dependent mechanism at the nuclear level of photoreceptor cells.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.exer.2015.10.020.

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